Premixes for Animal feeding stuffs

Determination of Ronozyme® RumiStar alpha-amylase activity

Contents

Introduction

This Method has been developed to quantify Ronozyme® RumiStar alpha-amylase in premixes. However, the method cannot be used to evaluate the in vivo efficacy of Ronozyme® RumiStar.

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1. Scope

This Method describes the determination of Ronozyme® RumiStar alpha-amylase activity in premix samples.
The method does not distinguish between Ronozyme® RumiStar alpha-amylase added as a feed additive and other alpha-amylases (e.g. endogenous alpha-amylase) already present in the premix materials.
The method cannot be used to evaluate or compare the *in vivo* efficacy of Ronozyme® RumiStar product. It is not a predictive method of the *in vivo* efficacy of amylases present on the market as they can develop different *in vivo* efficacy per unit of activity obtained with the method described.

The method is suitable and validated exclusively for the determination of Ronozyme® RumiStar alpha-amylase and exclusively in premixes.

NOTE 1 The method was developed on the basis of Ronozyme® RumiStar alpha-amylase. Therefore, it might not necessarily be suitable as such for alpha-amylase products presently on the market or which might be developed in the future. The method is thus a tool which is useful only to evaluate the total Ronozyme® RumiStar alpha-amylase in premix samples.

2. Terms and definitions

For the purposes of this document, the following terms and definitions apply.

alpha-Amylase unit (KNU)

One KNU is the amount of enzyme that releases in a two step reaction, Ronozyme® RumiStar / α-glucosidase, 6 µmol p-nitrophenol per minute from 1.86 mM ethylidene-G_7-p-nitrophenyl-maltoheptaoside at pH 7.0 and 37 °C.

3. Principle

Incubation of the substrate Red Starch with Ronozyme® RumiStar alpha-amylase depolymerizes the substrate by an *endo*-mechanism to produce low-molecular weight dyed fragments. The low-molecular weight dyed fragments remain in solution after addition of ethanol to the reaction mixture, whereas the high molecular weight material is precipitated. The high molecular weight material is removed by centrifugation and the clear colored supernatant is measured at 510 nm.
The alpha-amylase in the assay solution is quantified with an alpha-amylase standard curve made with a certified alpha-amylase standard for Ronozyme® RumiStar.

4. Chemicals and Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

WARNING This method requires the handling of hazardous substances. It is recommended to use various regulations for potentially hazardous chemicals. Organizational, technical and personal safety has to be observed.

4.1 Ronozyme® RumiStar standard (lot 97-12102; 103.9 KNU / g)

4.2 Hydrochloric acid 25%, p.a.; HCl

4.3 Sodium dihydrogen phosphate dihydrate, p.a.; $NaH_2PO_4 \cdot 2\ H_2O$

4.4 RED-Starch, (Megazyme S-RSTAR)

4.5 Imidazole, p.a.; $C_3H_4N_2$

4.6 Tween 20

4.7 Sodium hydroxide, p.a.; NaOH

4.8 Ethanol absolute p.a.; C_2H_5OH

4.9 EDTA (ethylenedinitrilotetraacetic acid disodiumsalt dehydrate); $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$

4.10 RED-Starch substrate solution, 1%, pH 5.0

Dissolve completely 2.0 g of RED-starch in approximately 160 ml distilled water (60°C to 65°C) and cool to room temperature. Add 20ml sodium phosphate buffer (4.14), adjust the pH to 5.0 with 25% hydrochloric acid and fill up to 200 ml with distilled water. Store in the refrigerator. The maximum storage time is 3 months.

4.11 Sodium hydroxide, 5M

Dissolve 100.0 g sodium hydroxide [NaOH] in approximately 400 ml distilled water and cool to room temperature. Fill up to 500 ml with distilled water. Store at room temperature. The maximum storage time is 6 months.

4.12 Tween 20, 10%

Dissolve 10.0 g of Tween 20 with distilled water and fill up to 100 ml. Store at room temperature. The maximum storage time is 6 months.

4.13 Imidazole buffer with 1.5% EDTA and 0.02% Tween 20, pH 8.0; 0.1 mol/l

Dissolve 34.0 g imidazole hydrochloride [$C_3H_4N_2$], 75.0 g EDTA [$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$] and 4.0 g sodium hydroxide [NaOH] in approximately 4,8 l distilled water. Adjust the pH to 8.00 ± 0.02 with 5M sodium hydroxide (4.11) and add 10 ml 10 % Tween 20 (4.12) Fill up to 5 l with distilled water. Store at room temperature. The maximum storage time is 2 weeks.

4.14 Sodium phosphate buffer with 0.02% Tween 20, pH 5.0; 0.1 mol/l

Dissolve 78.0 g sodium dihydrogen phosphate dihydrate [$NaH_2PO_4 \cdot 2\ H_2O$] in approximately 4.8 l distilled water. Adjust the pH with 5 M sodium hydroxide (4.11) to 5.00 ± 0.02 and add 10 ml 10% Tween 20 (4.12). Fill up to 5 l with distilled water. Store at room temperature. The maximum storage time is 2 weeks.

4.15 Sodium phosphate buffer, pH 5.0; 1.0 mol/l

Dissolve 78.0 g sodium dihydrogen phosphate dihydrate in approximately 450 ml distilled water. Adjust the pH with 5 M sodium hydroxide (4.11) to 5.00 ± 0.02 and fill up to 500 ml with distilled water. Store at room temperature. The maximum storage time is 2 weeks.

4.16 Ethanol STOP and PRECIPITATION reagent, 95%

50 ml distilled water are transferred into a 1 liter volumetric flask and filled up with ethanol absolute. Store at room temperature. The maximum storage time is 6 months.

4.17 Ronozyme® RumiStar stock standard solution

Weigh about 100.0 mg of Ronozyme® RumiStar standard Lot 97-12102 transfer it quantitatively to a 50 ml volumetric flask and dissolve it in 50 ml phosphate buffer with Tween 20 (4.14). Add a magnetic stir bar and stir for 30 – 60 minutes at room temperature. Calculate the exact concentrations of the Ronozyme® RumiStar stock standard solution. The maximum storage time is 1 day.

5. Apparatus

Usual laboratory apparatus, in particular, the following.

5.1 Water bath, thermostatically controlled (with inserts for 2 ml tubes).

5.2 Water bath, thermostatically controlled (with 4 remote controlled magnetic stirring systems – e.g. TELESYSTEM 06.40, VARIOMAG)

5.3 pH-meter, with two decimal digital readout.

5.4 Magnetic stirrers (≥ 20 W power)

5.5 Egg shaped stirring bars (40 mm x 20 mm)

5.6 Analytical balance, sensitivity 0.01 mg.

5.7 Balance, sensitivity 0.01 g.

5.8 Vortex mixer

5.9 Centrifuge for 2 ml microcentrifuge tubes, capable of 11 000-20 000 g.

5.10 Electronic dispenser

5.11 Pipettes (electronic and manual), in the range 10 µl to 2 000 µl.

5.12 Microplate spectrophotometer

5.13 Microcentrifuge tubes, 2 ml.

6. Sampling

A representative sample should be used. It should not have been damaged or changed during transport or storage. Sampling is not part of the method specified. A recommended sampling procedure is given in ISO 6497 [1].

7. Sample preparation

Two weighings are performed for each sample.

7.1 Premix samples

Two portions premix of about 5 g each, are weighed into 100 ml conical flasks. 100 ml imidazole buffer with Tween 20 (4.13) are added to the premix and the mixture is stirred (600 - 650 rpm) on a magnetic stirrer [5.4] for 60 minutes at room temperature. 2 ml of the premix extract are transferred to a tube (5.13)and centrifuged for 3 minutes in a centrifuge (11'000 to 20'000 g).

NOTE Inhomogeneity in the premix sample can lead to high CVs. For premix samples showing CVs > 15%: such inhomogeneity can derive from inhomogeneous particle size distribution in products or inhomogeneous premix preparation.

7.2 Dilution of Ronozyme® RumiStar extracts

Premix extracts have to be at least 10 fold diluted with phosphate buffer (4.14)

The extracts are diluted with phosphate buffer with Tween 20 (4.14) to a final concentration of 0.00025 $KNUml^{-1}$ to 0.00045 $KNUml^{-1}$.

Dilution example

The expected activity in a premix sample is 30'000 $KNUkg^{-1}$ and the final desired concentration is approximately 0.0003 $KNUml^{-1}$.

$$\text{Dilution factor} = \frac{30000\,KNU \times 0.005\,kg \times ml}{kg \times 100\,ml \times 0.0003\,KNU} = 5000$$

0.005 kg = weight of the premix sample; 100 ml = extraction volume

The feed extract should be 1:5000 diluted for the enzymatic reaction.

8. Procedure

8.1 Ronozyme® RumiStar standard

The Ronozyme® RumiStar stock standard solution (4.17) is diluted with phosphate buffer with Tween 20 (4.14) according to Table 1.

Table 1 — Dilution steps Ronozyme® RumiStar standard curve

Standard	Parts Stock Solution (4.16)	Parts Buffer (4.13)	Dilution Factor	KNU / ml[a]
A	1	249	250	0.00083
B	1	499	500	0.00042
C	1	749	750	0.00028
D	1	999	1000	0.00021

[a] The exact concentrations have to be calculated (4.17)

8.2 Ronozyme® RumiStar standard curve

For each sample blanks are included. For calculation of Ronozyme® RumiStar alpha-amylase activity, the blank values are subtracted from the sample values.
Triple determinations and two blanks are performed for each dilution. The procedure is described in Table 2.
Description of the assay: 200 µl diluted standard sample (8.1) are pipetted into a 2 ml tube (5.13). The sample is pre-incubated for 5 min at 40°C. 0.4 ml pre-heated (40°C) Red-Starch substrate (4.10) is added. The sample is mixed and incubated for exactly 60 min at 40°C. After 60 min 1 ml STOP and PRECIPITATION reagent (4.16) is added. The sample is mixed and kept for 15 min at room temperature. The sample is mixed again and then centrifuged for 3 min at 11 000 - 20 000 g. The clear supernatant is measured at 510 nm.
Blanks are NOT incubated at 40°C and the STOP and PRECIPITATION reagent (4.16) –STEP 1- is added prior to the Red-Starch substrate (4.10) –STEP 2.

8.3 Premix samples

Triple determinations and two blanks are performed for each extraction (7.1). The procedure is described in Table 2.

Description of the assay: 200 µl properly diluted premix extract (7.1 and 7.2) are pipetted into a 2 ml tube (5.13). The sample is pre-incubated for 5 min at 40°C. 0.4 ml pre-heated (40°C) Red-Starch substrate (4.10) is added. The sample is mixed and incubated for exactly 60 min at 40°C. After 60 min 1 ml STOP and PRECIPITATION reagent (4.16) is added. The sample is mixed and kept for 15 min at room temperature. The sample is mixed again and then centrifuged for 3 min at 11 000 - 20 000 g. The clear supernatant is measured at 510 nm.

Blanks are NOT incubated at 40°C and the STOP and PRECIPITATION reagent (4.16) –STEP 1- is added prior to the Red-Starch substrate (4.10) –STEP 2.

Table 2 — Procedure Ronozyme® RumiStar samples

Assay steps	Standard and samples	Blank
Diluted extract	200 µl	200 µl
Pre-incubation at 40 °C	5 min	NO
RED-Starch substrate, 40 °C (4.10)	0.4 ml	0.4 ml (STEP 2)
Mix	YES	YES
Incubation at 40 °C	60 min	NO
STOP and PRECIPITATION reagent (4.16)	1 ml	1 ml (STEP 1)
Mix	YES	YES
Room temperature	15 min	15 min
Mix	YES	YES
Centrifugation	3 min 11 000 – 20 000 g	3 min 11 000 – 20 000 g

9. Calculations

9.1 Formulation standard curve

A standard curve is made with the ΔOD 510 ($OD\ 510_{standard}$ – $OD\ 510_{blank}$) obtained with the Ronozyme® RumiStar standards (8.1 and 8.2) on the y-axis and the calculated alpha-amylase activity on the x-axis. The best fitting line is calculated by linear regression, y = *m*x, (set intercept at 0) as shown in Figure 1.

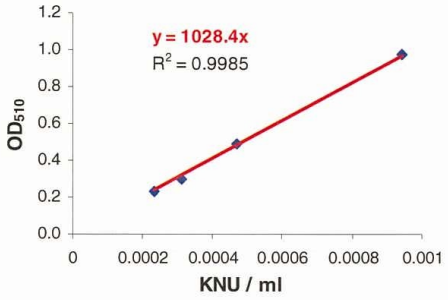

Figure 1 — Ronozyme® RumiStar standard (Lot 97-12102)

9.2 Calculation alpha-amylase activity

The alpha-amylase activity ($a_{\alpha\text{-amylase}}$) is calculated as follows:

$$a_{\alpha\text{-amylase}} = \frac{\Delta OD \times D}{m \times W}$$

where

ΔOD is $OD\ 510_{sample} - OD\ 510_{blank}$

m is the slope of the standard curve, in KNU^{-1} ml OD 510

D is the dilution factor (extraction volume x dilution of the extract), in ml

W is the weight of the sample, in kg

9.3 Examples

ΔOD = 0.372 OD_{510}

m = 1028.4 KNU^{-1} ml OD_{510}

D = 600 000 ml (100 ml extraction volume x 6000 [1:6000 dilution of extract])

W = 0.0052 kg

$$a = \frac{0.372 \times 600\,000}{1028.4 \times 0.005\,2} = 41'738\ KNU kg^{-1}$$

9.4 Remarks

- It is recommended to use nitrile gloves and plastic spoons when handling with imidazole hydrochloride.
- Components in premix extracts may alter the Ronozyme® RumiStar alpha-amylase activity. Therefore, **all premix extracts have to be at least 1:10 diluted with phosphate buffer (4.14)**.

10. Precision

10.1 Limit of Detection and Limit of Quantification

The determination of the Detection Limit ($L_D = 3\sigma$) and Quantification Limit ($L_Q = 10\sigma$) are according to the IUPAC nomenclature [1] and are given as ΔOD_{510} and KNU/kg.

Detection Limit L_D = 0,018 = 4 KNU / kg premix

Quantification Limit L_Q = 0,058 = 12 KNU / kg premix

10.2 Interlaboratory test

The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

10.3 Repeatability

The relative standard deviation of repeatability (RSD_r) is the average coefficient of variation from two independent results obtained from the same sample at the same day, from the same technician, with the same equipment and method.

The relative standard deviation of repeatability (RSD_r) is estimated to 4%.

10.4 Reproducibility

The relative standard deviation of reproducibility (RSD_R) is the average coefficient of variation from results obtained with the same sample using the same method, but measured at different days.

The relative standard deviation of reproducibility (RSD_R) is estimated to 4%.

Bibliography

[1] ISO 6497, Animal feeding stuffs — Sampling.

[2] Nomenclature in Evaluation of Analytical Methods Including Detection and Quantification Capabilities. Pure & Appl. Chem., Vol. 67, No. 10, pp. 1699-1723 (19